

## Creatinine Metabolism in *Cryptococcus neoformans* and *Cryptococcus bacillisporus*

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The pathogenic species of *Cryptococcus*, *C. neoformans* and *C. bacillisporus*, utilized creatinine as a source of nitrogen but not of carbon. Chromatographic and autoradiographic studies suggest that creatinine metabolism in both species involves a single step resulting in the production of methylhydantoin and ammonia. The enzyme responsible for this step, creatinine deiminase, was produced by the cells only in the presence of creatinine in both species. The synthesis of creatinine deiminase was repressed by ammonia in *C. neoformans*, but not in *C. bacillisporus*. A possible explanation for this variation, based on the ecological differences between the two species, is discussed. A novel method for measuring creatinine deiminase activity is also described.

The literature on creatinine metabolism in microorganisms, in general, is scanty, and none deals with fungi. In bacteria, most of the work was carried out in species of *Pseudomonas* (21, 22), *Arthrobacter* (11), and *Clostridium* (21). In *Pseudomonas* and *Arthrobacter* species, creatinine is converted to sarcosine via creatine and further to glycine (Fig. 1). Three different inducible enzymes involved in this pathway are found in *Pseudomonas putida* (22). There are two exceptions to this general metabolic pathway. In *Pseudomonas stutzeri*, creatinine is degraded to methylguanidine and acetic acid (23). Under anaerobic conditions, *Clostridium paraputrificum* dissimilates creatinine to *N*-methylhydantoin and ammonia (21) (Fig. 1).

Creatinine is one of the nitrogenous end products excreted by pigeons, and pigeon droppings are the best known natural reservoir of *Cryptococcus neoformans* (8). In 1962, Staib (20) found that creatinine is assimilated by *C. neoformans* but not by three other *Cryptococcus* species that he tested. Since then, it is widely accepted that pigeon droppings serve as a selective medium for the fungus in nature due to the high content of creatinine (1). However, survey shows that only two serotypes of *C. neoformans*, A and D, are isolated from pigeon droppings and not the B and C serotypes (4). The B and C serotypes have recently been described as a separate species, *C. bacillisporus* (14). In view of this difference, it is of considerable interest to see whether creatinine plays a decisive role in the ecology of the two species. To understand this question, we investigated the metabolic pathway of creatinine in *C. neoformans* and *C. bacillisporus*.

### MATERIALS AND METHODS

**Strains, media, and growth conditions.** The isolates, clinical or natural origin, that were used in this work were as follows: *C. neoformans*, serotype D: NIH 12 ( $\alpha$  mating type of type culture), 430, and 38-2; serotype A: NIH 272, 372, and 195; and *C. bacillisporus*, serotype C: NIH 191 ( $\alpha$  mating type of type culture); serotype B: NIH 444 and 409. The growth medium was creatinine-glucose broth containing creatinine, 5 g; glucose, 20 g (unless stated otherwise);  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1 g;  $\text{KH}_2\text{PO}_4$ , 1 g; thiamine-vitamin solution (Bejectal, Abbott Laboratories, Chicago, Ill.), 50  $\mu\text{l/liter}$ . In some experiments, 3 g of ammonium sulfate, arginine, sodium glutamate, or potassium aspartate per liter was used with or without creatinine as a nitrogen source. When ammonium sulfate was used, 30 g of  $\text{KH}_2\text{PO}_4$  per liter was added to maintain the pH. All the isolates were grown at 25°C on a shaker (200 rpm). The inoculum was started from a malt agar slant. Cells collected from a slant were transferred into 50 ml of medium and incubated for 15 h. The growth rate of this culture was calculated by measuring the optical density at 600 nm. Cells were washed twice with fresh medium and transferred into 80 ml of medium in an amount estimated to produce, after approximately 18 h, an exponential culture yielding  $2 \times 10^7$  to  $3 \times 10^7$  cells per ml (0.4 to 0.6 optical density at 600 nm, Gilford spectrophotometer 240). This procedure is critical since the cells synthesize a thick polysaccharide capsule during the stationary phase, which interferes with the absorbance measurement and lowers the rate of cell breakage by the method described below.

**Preparation of cell-free extract.** The cells were harvested by centrifugation at  $12,000 \times g$  for 10 min in the cold and washed once with 0.1 M phosphate buffer, pH 8, containing 0.02% azide as preservative. The cells were then resuspended in the same buffer to give a concentration of 1 g/ml (wet weight). The yeast cells

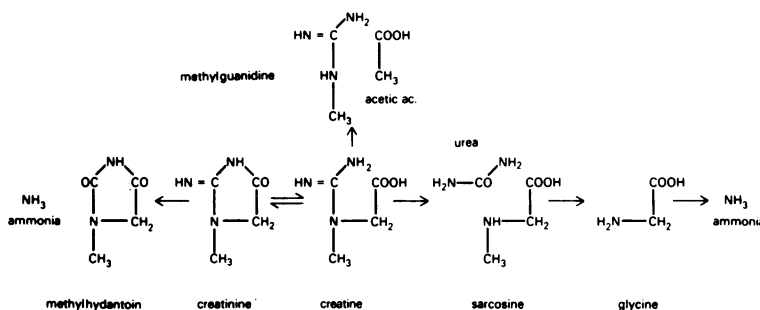


FIG. 1. Pathways of creatinine metabolism in bacteria.

were broken by shaking with glass beads. A 3.5-g amount of acid-washed, cold glass beads (0.45 to 0.50 mm, B. Braun, Melsungen) was added to 2 ml of cell suspension in a 15-ml conical graduated screw-capped plastic tube (Falcon, Oxnard, Calif.). The cells were broken by rapid mixing on a Vortex mixer (16). The glass bead treatment was carried on for periods of 15 s followed by 15-s cooling intervals until only broken cells were seen with cotton-blue staining under a microscope. It required a minimum 5-min treatment to obtain 99% loss of viability as estimated by colony counts. Cell-free supernatants were obtained by centrifugation at  $15,000 \times g$  for 15 min in the cold. The cell-free extracts were then stored at  $-18^\circ\text{C}$ . Cell wall and membrane fractions were separated by differential centrifugations as previously described (19).

**Assay of creatinine deiminase.** Creatinine deiminase (EC 3.5.4.21) activity was generally measured by the following procedure: 5 to 25  $\mu\text{l}$  of cell-free extract was incubated with 50 mM phosphate buffer (pH 8), 20 mM creatinine (Sigma, St. Louis, Mo.), and 5  $\mu\text{Ci}$  of either [ $^{14}\text{C}$ -carbonyl]creatinine (specific activity, 3.5 mCi/mmol; California Binuclear Corp., Sun Valley, Calif.) or [ $^{14}\text{C}$ -methyl]creatinine (specific activity, 5.3 mCi/mmol; California Binuclear Corp.) per ml in a final volume of 50  $\mu\text{l}$ . The mixture was incubated for 30 min at  $37^\circ\text{C}$ , and the reaction was stopped with 5  $\mu\text{l}$  of concentrated acetic acid. Then, 200  $\mu\text{l}$  of aqueous suspension of the cation-exchange resin AG 50W-X8 (1 g/ml; Bio-Rad, Richmond, Calif.) in the hydrogen form was added to the above mixtures. After shaking on Thomas-Boerner vertical shaker for 15 min, the suspensions were filtered into scintillation vials through Gelman A/E glass fiber filters by using a 10-place filter holder (Bio-Rad). The tubes and the filters were washed three times with 0.2-ml portions of water. To the combined filtrate, 15 ml of Aquasol (New England Nuclear, Boston, Mass.) was added for scintillation counting. One unit of enzyme was defined as the amount which catalyzed the decomposition of 1 nmol of creatinine per min under the assay conditions. The specific activity was expressed as units of activity per milligram of protein.

**Analytical methods.** Creatinine was estimated by Jaffé's reaction as described by Folin (10) and modified by Tsuru et al. (21) for creatininase assay. Creatinine was determined by the method of Eggleston et al. (7) as modified by Ennor and Stocken (9). Ammonia was measured either by Conway's microdiffusion technique followed by nesslerization (14) or by an enzy-

matic method which was based on the reductive amination of  $\alpha$ -ketoglutarate with glutamate dehydrogenase and NADH (Sigma technical bulletin no. 170-UV). Methylhydantoin was measured by the radioactive method described for the enzyme assay. Protein was determined by Bio-Rad protein assay (bulletin 1069, Bio-Rad) with bovine gamma globulin as a standard.

**Thin-layer chromatography and autoradiography.** Creatinine metabolites were separated by thin-layer chromatography on linear K silica gel (4.0 nm; type LK-6; Whatman Inc., Clifton, N.J.) with phenol-ethanol-water (14:4:1) as the solvent. After 6 h at room temperature, the spots were located with alkaline picric acid before and after heating at  $115^\circ\text{C}$  (15) and with ninhydrin reagent. Detection of labeled compounds on the thin-layer chromatogram was carried out by autoradiography with the use of X-Omat R film (XR-5; Eastman Kodak Co., Rochester, N.Y.). The exposure time was approximately 15 h at room temperature.

## RESULTS

**Growth in creatinine medium.** No growth of any isolate was observed in minimal medium containing creatinine as a sole source of both nitrogen and carbon. Isolates of *C. neoformans* and *C. bacillisporus* used creatinine only as a source of nitrogen but not of carbon. Creatine, on the other hand, was not utilized as a source of nitrogen or carbon in either species. All the isolates of *C. bacillisporus* and serotype D of *C. neoformans* utilized creatinine as readily as ammonium sulfate (optical density 0.38 in 18 h). Isolates of *C. neoformans* serotype A were of two kinds: NIH 272 utilized creatinine as well as ammonia (5), whereas NIH 372 and NIH 195 utilized creatinine so poorly that the similar growth was detected only after 4 days. These serotype A isolates were selected for their disparity in creatinine utilization (14) and were found to have an inefficient uptake mechanism for creatinine (unpublished data).

**Pathway of creatinine decomposition.** To determine the degradation pathway of creatinine, it was necessary to identify the metabolites. Thin-layer chromatography and autoradiography were utilized for this purpose. The solvent

system used (phenol-ethanol-water) yielded very good separation of the known metabolites of creatinine (Fig. 1) on the silica gel chromatogram. Figure 2 represents an experiment where radiolabeled creatinine was incubated with the cell-free extract and then chromatographed. The autoradiograms showed that after 30 min, the disappearance of creatinine was associated with the accumulation of only one product. No other labeled compound was found on the chromatogram, except for the unused creatinine. After 24 h of incubation, all of the radioactivity was located in one spot. Identical results were obtained by using [ $^{14}\text{C}$ ]creatinine labeled on either the carbonyl group or the methyl group. The chromatogram indicated that the product was *N*-methylhydantoin. This result was confirmed by using other solvent systems (butanol-pyridine-water, 1:1:1; methanol-pyridine-water, 20:1:5) and by chemical or physical analyses including UV spectra, HCl hydrolysis, and development of characteristic color with alkaline picrate (21).

The chromatographic studies indicated that creatinine metabolism in the cryptococci involved a single step resulting in production of methylhydantoin and ammonia (see Fig. 1). This conclusion was confirmed by a quantitative measurement of the conversion of creatinine to methylhydantoin and ammonia. The amount of creatinine which disappeared during the incubation with the cell-free extract was in equimolar ratio to the amounts of methylhydantoin and ammonia produced.

**Detection of creatinine-decomposing enzyme and some of its properties.** The enzyme responsible for creatinine metabolism in the cryptococci, creatinine deiminase, showed very low affinity for creatinine. The standard assay for the enzyme is based on a method of measuring creatinine by Jaffé's reaction which permitted the use of a maximum concentration of 0.5 mM creatinine in the enzyme reaction mixture. Therefore, it was very important to establish a new assay which did not depend on substrate concentration. The finding that creatinine was converted to methylhydantoin in the cryptococci made it possible to develop a very sensitive radioactive assay for the enzyme involved in the process.

The method was based on the fact that by decreasing the pH of the reaction mixture to pH 3.5, creatinine became positively charged, whereas methylhydantoin remained neutral (20). Any remaining radiolabeled substrate, after the incubation, was eliminated by adsorbing on cation-exchange resin (Dowex-50- $\text{H}^+$ ) and then filtering the suspension. Both the resin and the creatinine remained on the filter, whereas the methylhydantoin was collected in the filtrate

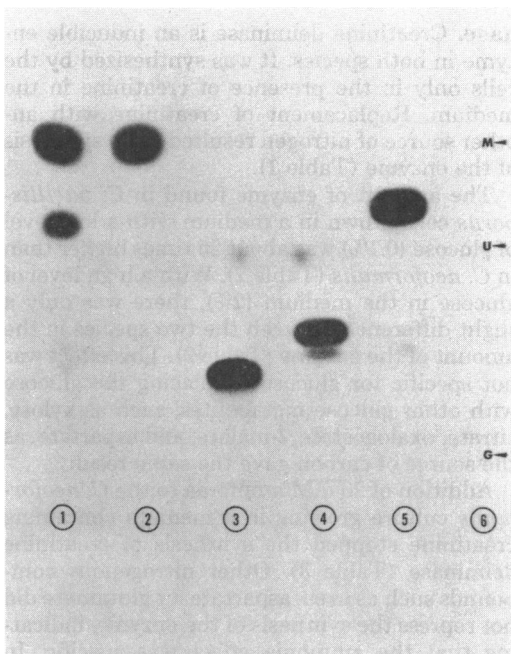


FIG. 2. Autoradiograph of creatinine metabolites of *C. neoformans* separated by thin-layer chromatography. Lane 1, 30-min incubation of cell-free extract with [ $^{14}\text{C}$ ]creatinine. Lane 2, 24-h incubation of the same reaction mixture as above. Lane 3, [ $^{14}\text{C}$ -carboxyl]sarcosine. Lane 4, [ $^{14}\text{C}$ -carboxyl]creatinine. Lane 5, [ $^{14}\text{C}$ -carbonyl]creatinine. Lane 6, arrows indicate the locations of non-radiolabeled standards: M, methylhydantoin; U, urea; G, glycine. The same results were obtained from *C. bacillisporus*.

and counted. Chromatographic study confirmed that all the radioactivity found in the filtrate resulted from radiolabeled methylhydantoin produced from the creatinine.

Creatinine deiminase had a broad optimum pH around 8 (data not shown). The optimum temperature was 37°C, with maximum ranging between 30 and 40°C. The rate of the reaction was proportional to the concentration of the enzyme up to 4 U/ml and gave linearity with incubation time. The activity of the enzyme was not affected by a chelating compound (EDTA), reducing agent (2-mercaptoethanol), and various metals. The enzyme showed very low affinity for the creatinine. The apparent  $K_m$  calculated from a Lineweaver-Burk plot was 14.3 mM. Creatine could not replace creatinine as the substrate of the enzyme. The enzyme was found only in the soluble fraction after cell breakage. Enzyme activity was detected in neither the culture medium nor the cell wall nor the membrane fractions.

**Effect of creatinine, ammonia, and glucose on the synthesis of creatinine deimi-**

nase. Creatinine deiminase is an inducible enzyme in both species. It was synthesized by the cells only in the presence of creatinine in the medium. Replacement of creatinine with another source of nitrogen resulted in no synthesis of the enzyme (Table 1).

The amount of enzyme found in *C. bacillisporus* cells grown in a medium with a low level of glucose (0.1%) was about 25 times higher than in *C. neoformans* (Table 2). With a high level of glucose in the medium (2%), there was only a slight difference between the two species in the amount of the enzyme (Table 2). This effect was not specific for glucose. Replacing the glucose with other glucose metabolites, such as xylose, citrate, oxaloacetate, *l*-malate, and aspartate, as the source of carbon gave the same result.

Addition of 20 mM ammonia to the *C. neoformans* culture growing in a medium containing creatinine stopped the synthesis of creatinine deiminase (Table 3). Other nitrogenous compounds such as urea, aspartate, or glutamate did not repress the synthesis of the enzyme, indicating that the ammonia effect was specific. In contrast to *C. neoformans*, ammonia did not repress the synthesis of the enzyme in *C. bacillisporus* (Table 3). Ammonia and methylhydantoin up to 90 mM did not inhibit creatinine deiminase activity in either species (data not shown).

## DISCUSSION

In contrast to some species of *Pseudomonas* (22, 23) and *Arthrobacter* (11), creatinine metabolism in the cryptococci involves only a single step, the production of methylhydantoin and ammonia. This explains why the two species of *Cryptococcus* did not utilize creatinine as a carbon source and used it only to obtain nitrogen

TABLE 1. Induction of creatinine deiminase in *C. neoformans* and *C. bacillisporus*

N source <sup>a</sup>	<i>C. neoformans</i> (NIH 12)		<i>C. bacillisporus</i> (NIH 191)	
	Total enzyme <sup>b</sup>	Sp act (U/mg of protein)	Total enzyme	Sp act (U/mg of protein)
Ammonia	0.58	0.32	0.50	0.28
Glutamic acid	5.36	1.48	2.18	0.61
Aspartic acid	2.72	0.15	1.47	0.06
Arginine	3.58	2.18	3.38	2.19
Creatinine	97.30	58.40	104.00	58.30

<sup>a</sup> The inoculum was grown in glucose broth with ammonium sulfate, washed twice, and then transferred into the media with the N sources listed above.

<sup>b</sup> Enzyme units in cell-free extract obtained from ca. 10<sup>10</sup> cells.

TABLE 2. Effect of glucose level on creatinine deiminase synthesis

Species	Glucose concn (%)	Creatinine deiminase	
		Total enzyme <sup>a</sup>	Sp act (U/mg of protein)
<i>C. neoformans</i> (NIH 12)	2.0	136.7	73.3
(serotype D)	0.1	6.0	1.8
<i>C. bacillisporus</i> (NIH 191) (serotype C)	2.0	180.0	105.0
	0.1	156.7	86.7

<sup>a</sup> Enzyme units in cell-free extracts obtained from ca. 10<sup>10</sup> cells.

TABLE 3. Ammonia repression on the synthesis of creatinine deiminase

N source <sup>a</sup>	Creatinine deiminase <sup>b</sup>	
	<i>C. neoformans</i> (NIH 12)	<i>C. bacillisporus</i> (NIH 191)
Creatinine	89.7	69.2
Ammonia-creatinine	3.5	63.2
Glutamic acid-creatinine	81.6	62.5
Aspartic acid-creatinine	71.2	56.3

<sup>a</sup> The inoculum was grown in creatinine-glucose broth, washed twice, and then transferred into the media with each of the N sources.

<sup>b</sup> Specific activity (enzyme units per milligram of protein).

from creatinine. This type of metabolism is the fastest and the most economical way to supply all of the nitrogen required. Among the bacteria, only *Clostridium paraputrificum* has been shown to have a similar creatinine metabolism with the exception that the bacterium could use creatine as well as creatinine as a source of nitrogen (21).

The synthesis of creatinine deiminase in *C. neoformans* was subjected to two types of regulation. It was induced by creatinine and repressed by ammonia. In contrast, the synthesis of this enzyme in *C. bacillisporus* was only under creatinine induction but not controlled by ammonia. These observations suggested that the natural reservoir of *C. bacillisporus* does not contain ammonia and creatinine at the same time. For this species, creatinine induction was sufficient for regulation and synthesis of creatinine deiminase.

Ammonia repression of the synthesis of enzymes involved in nitrogen utilization is of widespread occurrence in the fungi. It was reported for purine degradation (2) and for arginine, proline, and histidine catabolism (3, 17). Genetic studies in *Aspergillus nidulans* (2, 3, 18) sug-

gested that ammonia repression is under positive control. A gene product essential for the synthesis of large numbers of enzymes in nitrogen metabolism could not function in the presence of ammonia (2, 17). Avian droppings are rich in ammonia (24); hence ammonia repression in *C. neoformans* may be an adaptive control mechanism which was not developed in *C. bacillisporus*. Metabolic regulation by ammonia in *C. neoformans* may save energy and substances needed for the synthesis of creatinine deiminase and possibly for other enzymes involved in nitrogen metabolism (17). Although two of the three *C. neoformans* serotype A isolates (NIH 372 and 195) grew very slowly in the medium containing creatinine as a sole source of nitrogen (5), the regulation of creatinine deiminase synthesis in these isolates was the same as that in the serotype D isolate.

The amount of creatinine deiminase was very small when isolates of *C. neoformans* were grown in a low concentration of glucose, in contrast with the situations known in other fungi. Lowering the glucose concentration in the media usually derepresses the synthesis of the enzymes involved in nitrogen metabolism in other fungi (2, 6, 17). In *C. neoformans*, the glucose effect was probably an indirect effect. It was related to the total metabolism of the fungus, which was dictated by the availability of glucose. At high levels of glucose, all of the ammonia produced by creatinine deiminase was utilized by the fungus. When the glucose level in the medium decreased, the synthesis of the carbon-containing nitrogenous compounds ceased. As a consequence, ammonia accumulated (14). In *C. neoformans*, the amount of accumulated ammonia was enough to repress further synthesis of the creatinine deiminase, whereas in *C. bacillisporus* enzyme synthesis and production of ammonia were uninterrupted. This finding may explain the biochemical basis of the newly developed diagnostic test (creatinine-glucose-bromothymol blue agar) for the identification of the two species (14).

The similarity in creatinine metabolism by *C. bacillisporus* and *C. neoformans* eliminates the possibility that creatinine plays a decisive role for their ecological differences. However, the data presented here suggest that only *C. neoformans* has evolved a specific regulatory mechanism of creatinine metabolism to suit the conditions of pigeon droppings. This, in turn, may explain that pigeon droppings are a natural reservoir rather than a random source for *C. neoformans*. This difference between the B,C and A,D serotypes also supports the view that we are dealing with two different species of *Cryp-*

*tococcus*. More fundamental differences between B,C and A,D were already published (4, 5, 12-14).

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